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14. ABSTRACT

We have made gradual progress in each aim. In aim 1, we have identified TA p63 as the dominant isoform upregulated by Sox2. We then performed a chromatin immunoprecipitation using a Sox2 antibody followed by sequencing that did not identify p63 as a direct target of Sox2. Despite this finding, the sequencing data demonstrate several intriguing targets that we have begun to study. These include the protein tyrosine phosphatase zeta 1 (Ptprz1) and connective tissue growth factor (CTGF). In addition for this aim, we have re-derived and bred a p63 conditional knockout mouse to our conditional Sox2-overexpressing mouse. Over the next year, these mice will document the importance of p63 for Sox2-induced oncogenesis. For aim 2, we have performed initial transplants but need to troubleshoot the protocol to add supportive fibroblasts. For aim 3, we are breeding mice to pure strains in order to identify modifier genes that incidence of tumors after Sox2 overexpression.

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Progress Report: The Mechanism of Sox2-Induced Lung Cancer

October 29, 2011

Introduction

The purpose of the proposed research is to clarify the mechanism of Sox2-induced non-small cell lung cancer. The first aim involves investigating p63 as a direct target of Sox2 in tumor cells. The second aim investigates the tumor-initiating ability of proximal and distal respiratory epithelial cells when Sox2 is overexpressed. The third aim tests the hypothesis that strain-specific modifier genes affect the phenotype of mice inducibly overexpressing Sox2 in Scgb1a1-expressing cells. We have begun work on all three aims.

Aim 1

We have made gradual and steady progress toward achieving the work proposed in Aim1 of the grant. The main purpose of this aim is to identify the importance of the p63 gene in Sox2-induced oncogenesis. Because p63 has been demonstrated to be important in the maintenance of squamous epithelia, because it leads to squamous metaplasia when overexpressed in the mouse, and because p63 is expressessed in SOX2+ human squamous lung cancers and in Sox2+ basal cells, we hypothesized that p63 is a direct target of Sox2 and that it is essential in Sox2's ability to intiaite lung cancer.

Because p63 is expressed in 2 different isoforms, we first developed primers for each isoform. Reverse transcription-polymerase chain reaction (RT-PCR) was carried out using these primers, and the results are depicted in Figure 1. As the figure demonstrates, the TA-p63 isoform is upregulated by Sox2 in our Scgb1a1-CreER-induced mouse model. The delta-N isoform is not detected.

The next goal was to perform chromatin immunoprecipitation (ChIP) using an antibody for Sox2. However, the small number of cells recovered per mouse (using elastase digestion of the intraparenchymal lung epithelium followed by sorting for GFP) made performance of ChIP from these cells impractical. We thus changed course to use the SOX2-overexpressing human squamous cell lung carcinoma cell lines H520 and 2170. Expansion of these cells gave us enough material for efficient ChIP. After the cells were fixed and sonicated to shear the DNA, immunoprecipitation was performed using the Seven Hills Bioreagents Sox2 antibody. Crosslinking was then reversed, and massively parallel sequencing was carried out using the Duke IGSP Genome Sequencing and Analysis Core. Analysis of these sequence reads surprisingly demonstrated that p63 is not a direct target of SOX2. Thus, it may be that p63 is being upregulated by another direct target of SOX2. Correlation analysis of p63 and

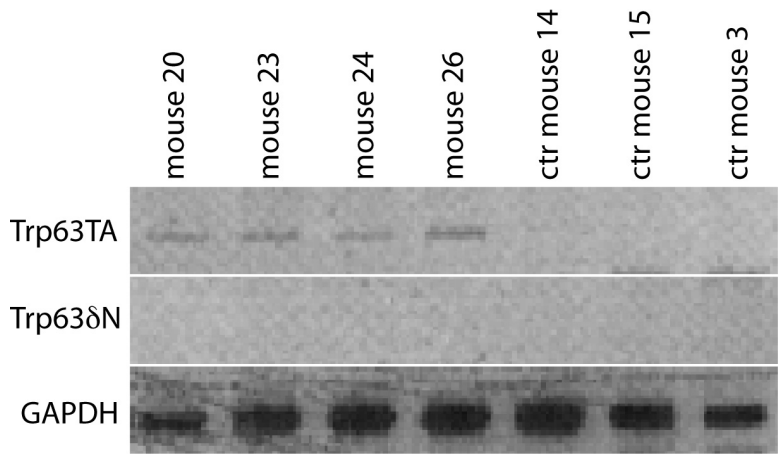

Figure 1: RT-PCR of p63 isoforms in control Scgb1a1-CreER; farnesylated green fluorescent protein (fGFP) cells and Scgb1a1-CreER; lox-stop-lox (lsl) Sox2-IRES-GFP cells

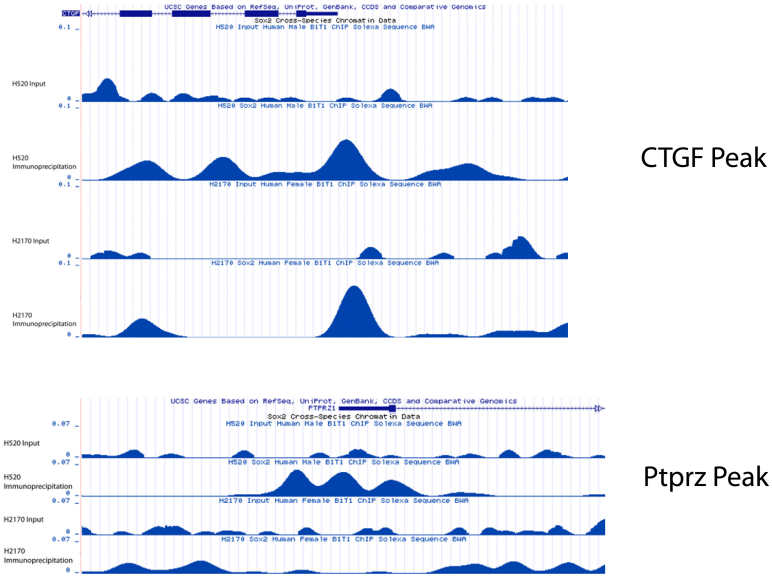

Figure 2: Examples of sequencing data from the ChIP experiment. Top: CTGF locus; Bottom: Ptprz locus

DIrect SOX2 Targets

Gene	Fold-change in mouse tumors
PTPRZ1	62.6
CTGF	35.6
KRT20	27.5
PAX9	23.6
ITGA6	18.0
KLF5	11.3

Table 1: Table 1: Abbreviated list of direct targets from Sox2 ChIP-seq that are also upregulated in mouse Sox2-induced tumors

other SOX2-upregulated genes across SOX2-positive human lung tumors may identify potential candidates (genes that vary in a similar pattern to p63). Once potential genes are identified, we will see if they were identified in the SOX2-ChIP. We will order lentiviral shRNAs against these targets, infect H520 cells, and perform RT-PCR to asertain whether p63 is downregulated.

Although p63 was revealed not to be a direct target of SOX2, the ChIP-seq is very valuable in that it identified several interesting genes that are direct targets of SOX2 in human lung cancer cells. We compared these genes to microarray data obtained from our mouse model. Genes that were present in the sequencing data and transcripts that were significantly upregulated in Scgb1a1-CreER; lsl Sox2-IRES GFP cells in comparison with Scgb1a1-CreER; lsl fGFP cells were identified. A sampling of this list of these genes is included as Table 1. We have begun to characterize some of these genes. Among them are Protein tyrosine phosphatase receptor-zeta 1 (Ptprz1) and connective tissue growth factor (CTGF) (Figure 2). Each of these has been liked to oncogenesis in various contexts. We have infected H520 cells with lentiviral siRNA specific to each of these genes and seen decreased cell viability that is not present in cells infected with lentiviral scrambled control siRNA. We are presently transplanting these siRNA-expressing cells into Rag1-/- mice subcutaneously in order to assess effects on tumor formation.

The next item in the Statement of Work involves breeding an inducible p63-knockout allele into the Scgb1a1-CreER; Rosa26-Sox2-IRES GFP mouse line to assess the necessity of p63 for Sox2-induced tumorigenesis. Our close collaborator Barry Stripp has just finished re-deriving the inducible p63-knockout (floxed p63) mouse line from frozen embryos. We have obtained this mouse line and crossed it to our mice. We have finally obtained a litter of mice that contain all necessary transgenes in a homozygous fashion. Controls will be littermate controls that are heterozygous for wild-type and floxed p63. This litter will be injected with tamoxifen at the beginning of November, and more litters are planned. These experiments will directly evaluate the role of p63 in Sox2-induced tumorigenesis.

Aim 2

The main goal of Aim 2 is to explore whether there is a microenvironmental contribution to Sox2-induced oncogenesis in the mouse. We planned to isolate proximal Scgb1a1-CreER; lsl Sox2 IRES GFP cells via flow sorting for transplantation into the retroorbital veins of immunodeficient Rag1-/- mice. While our preliminary data demonstrated that this transplantation led to tumors when the transplant was performed using K-RasG12D-expressing cells, we have not obtained tumors using the Sox2-overexpressing proximal cells. Possible explanations for this lack of tumor formation include lack of ability of the proximal cells to form tumors no matter the environment as well as a possible need for co-transplantation with stromal cells. However, transplants of distal epithelial cells have also not grown robustly. We have reasoned that the transplanted cells may require fibroblasts for support. We have thus initiated transplants using mouse fibroblasts 1:1 with Sox2-overexpressing proximal and distal Clara cells. Evaluation of the recipient mouse lungs for tumor formation is imminent. In addition, it may be that tumor cells lodging in capillaries does not adequately recapitulated the alveolar microenvironment. We have thus begun to explore intratracheal transplantation as well. Over the next 2-3 months, we should have the troubleshooting of these transplants completed so that we can evaluate the effect of the local microenvironment on Sox2-induced tumor formation. Once the transplants are successful, we will analyze tumors that form as Statement of Work 2b and 2c state.

Aim 3

The final aim of the grant is to explore the role of modifier genes in Sox2-induced neoplasia. Approximately half of the Scgb1a1-CreER; Rosa26-Sox2-IRES GFP mice develop cancer on a mixed 129/C57/Bl6 background. We propose to breed our mixed mouse line to pure C57/Bl6, 129, and AJ backgrounds. The crosses have begun in our animal facility. We are still collecting information about the speed genetics programs at various vendors. After a few generations, we will seek input from a vendor about which mice to breed to achieve pure strains of Scgb1a1-CreER; Rosa26-Sox2-IRES GFP mice. Once we have pure lines, the genomic analyses stated in SOW task 3 will be carried out.

KEY RESEARCH ACCOMPLISHMENTS

- Determination of the isoform of p63 induced by Sox2 overexpression
- Identification of direct SOX2 transcriptional targets by ChIP-sequencing

REPORTABLE OUTCOMES

No reportable outcomes yet. We envision at least 2 manuscripts emanating from the p63 conditional knockouts and from nove ChIP-sequencing targets.

CONCLUSION

We are steadily identifying key mechanistic components downstream of Sox2. Although p63 seems not to be a direct transcriptional target, the conditional knockout will at least delineate its importance in oncogenesis downstream of Sox2 upregulation. Several other interesting genes have been identified when comparing the microarray of the mouse tumor samples and the human tumor cell line SOX2 ChIP-sequencing. Work on the importance of tumor microenvironment and potential strain-specific modifier genes is ongoing.

We expect that these studies will increase our understanding of how Sox2 leads to cancer formation. Potentially, such information will lead to targeted therapeutics that would be useful in treating SOX2-positive human lung cancers as we have a paucity of agents that work well in squamous cell lung cancer.